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ABSTRACT **During a search for genes controlling conidial dormancy in *Aspergillus fumigatus*, two dehydrin-like genes, *DprA* and *DprB*, were identified. The deduced proteins had repeated stretches of 23 amino acids that contained a conserved dehydrin-like protein (DPR) motif. Disrupted *DprA*Δ mutants were hypersensitive to oxidative stress and to phagocytic killing, whereas *DprB*Δ mutants were impaired in osmotic and pH stress responses. However, no effect was observed on their pathogenicity in our experimental models of invasive aspergillosis. Molecular dissection of the signaling pathways acting upstream showed that expression of *DprA* was dependent on the stress-activated kinase SakA and the cyclic AMP-protein kinase A (cAMP-PKA) pathways, which activate the bZIP transcription factor AtfA, while expression of *DprB* was dependent on the SakA mitogen-activated protein kinase (MAPK) pathway, and the zinc finger transcription factor PacC. Fluorescent protein fusions showed that both proteins were associated with peroxisomes and the cytosol. Accordingly, DprA and DprB were important for peroxisome function. Our findings reveal a novel family of stress-protective proteins in *A. fumigatus* and, potentially, in filamentous ascomycetes.**

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INTRODUCTION

The human pathogen *Aspergillus fumigatus* has the remarkable ability to survive extended periods of adverse environmental conditions. This filamentous fungus produces large amounts of dormant asexual spores (conidia). Dormant conidia exhibit resistance to a variety of environmental stresses, including desiccation, extreme temperatures, and osmotic or oxidative stress. When favorable conditions occur, these metabolically inactive cell types will reactivate the cell biochemical and genetic machineries, and alleviate resistance-associated functions. It is expected that the expression of genes involved in the establishment of conidial dormancy would be down-regulated during germination. Indeed, transcriptomics studies initiated at a time the genome was not yet sequenced indicated that around one-fourth of the *A. fumigatus* genes had transcripts in the dormant conidia, and that the expression of 22% of these genes was down-regulated during germination (Lamarre *et al.*, 2008), leading to the hypothesis that at least some of these genes would be essential for the establishment and control of conidial dormancy. Using this strategy led us to identify Afu4g00860, a gene constitutively transcribed in dormant conidia, the expression of which was down-regulated within 30 min in yeast extract peptone-dextrose (YPD; Lamarre *et al.*, 2008). This gene codes for a protein of unknown function harboring five repeated domains and was called *DprA* based on the amino acid repeats. Another gene, encoding nine such domains, was also found in the genome of *A. fumigatus* (*DprB*: Afu6g12180). This study reports on the functional analysis of *DprA* and *DprB*. Phenotype analysis of disruption mutants indicated the involvement of DprA and DprB in specific stress responses. The use of signal transduction mutants in

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Abbreviations used: cAMP, cyclic adenosine monophosphate; DPR, dehydrin-like protein; DTT, dithiothreitol; IUP, intrinsically unstructured protein; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PTS, peroxisome targeting signal; YPD, yeast extract peptone-dextrose.

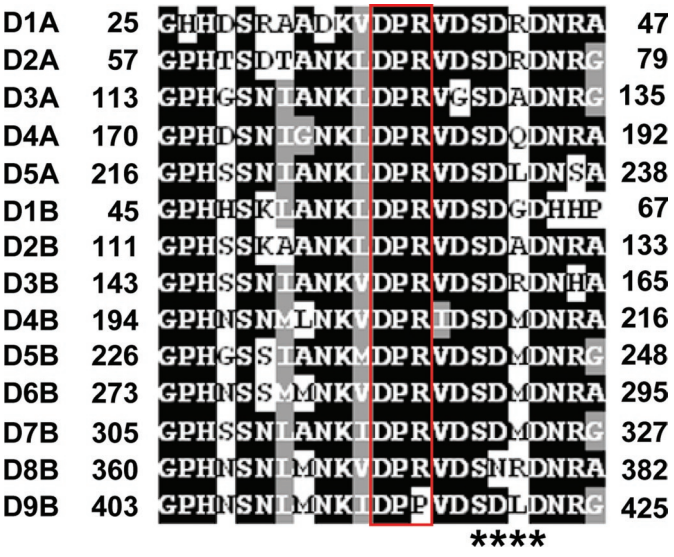

FIGURE 1: Alignment of the DPR domains from DprA and DprB. Conserved amino acids are boxed in black (identical) or gray (similar). D1A-D5A designate the five domains from DprA and D1B-D9B refer to the nine domains from DprB, numbered from N- to C-terminal end. Numbers indicate the amino acid positions. The DPR motif is indicated with a red border. Asterisks designate a predicted phosphorylation site conserved in all DPR domains.

monitoring *Dpr* expression levels suggested convergence of several pathways on the regulation of *Dpr* genes. Subcellular localization revealed that both proteins were associated with the cytosol and peroxisomes. This study uncovers novel proteins involved in stress protection in *A. fumigatus*.

RESULTS

DprA (Afu4g00860) and *DprB* (Afu6g12180) encode fungal dehydrin-like proteins

The gene Afu4g00860 was retrieved from an expression profiling study conducted in *A. fumigatus* aiming at the identification of germination-regulated genes (Lamarre *et al.*, 2008). The deduced sequence corresponded to a protein containing 246 amino acids. Scanning of the sequence by prediction software neither revealed known localization signatures nor gave indications of protein function. However, blast results gave a hit with a 435-residue protein from *A. fumigatus*, encoded by Afu6g12180. Remarkably, blast results aligned only on small portions of the proteins, with very poor homology in between. The homologous sequences were repeated five and nine times, respectively, and corresponded to the signature pattern of fungal dehydrins (Abba *et al.*, 2006). The repeated domain consisted of a stretch of 23 amino acids, containing a conserved dehydrin-like protein (DPR) motif (Figure 1). For this reason, the genes were called *Dpr*. Dehydrins were described in plants, where they are involved in the protection against dehydration-related stresses (Rorat, 2006). However, they have not been studied in fungi. In silico analysis confirmed that DprA and DprB were dehydrin-like proteins, by virtue of their physicochemical properties (Wise, 2003; Abba *et al.*, 2006; Supplemental Figure S1). The presence of hydrophobic residues, predicted phosphorylation sites, and proline residues within the DPR domains (Figures 1 and S1B) suggest that DPR domains could form a hydrophobic core, within which protein–protein interactions would take place.

DprA and *DprB* are down-regulated upon conidial germination

Expression of *DprA* and *DprB* was assessed during conidial germination (Figure 2A). In dormant conidia (time 0), *DprA* transcripts were ~120 times more abundant than *DprB*. Both transcript types underwent significant down-regulation with a 1000- and a 350-fold decrease in expression level from 0 to 30 min, respectively. Very weak expression of *DprA* was detected beyond 30 min, at least up to 24 h. In contrast, *DprB* transcripts became abundant again after 8 h and reached a twofold increase at 24 h.

To monitor the expression during development, the coding sequences of *DprA* and *DprB* were fused to *eGfp* under the control of their own promoters. DprA-eGfp fluorescence was detected only in dormant conidia (Figure 2B). In agreement with the real-time PCR data, DprB-eGfp fluorescence was observed in dormant conidia. However, in contrast to DprA-eGfp, DprB-eGfp was also observed in the conidiophores (Figure 2C) and in hyphae (unpublished data), indicating DprB was associated with late stages of development, unlike DprA.

DprA is involved in the oxidative stress response of conidia

Growth of *DprA*Δ (but not *DprB*Δ) mutants was inhibited by oxidative stress generated by hydrogen peroxide or paraquat at concentrations > 2 mM (Figure 3A). Consistent with this, *DprA* expression was up-regulated upon treatment with 2 mM H_2O_2 or 2 mM paraquat (Figure 3B). In the absence of stress, no difference was observed between the germination curves of the mutant and control strains (Figure 3C). However, when 2 mM H_2O_2 was added to the medium, germination of the *DprA*Δ (and *DprA*Δ *DprB*Δ) mutants was impaired (Figure 3D). After 13 h, germination had reached its maximum stage. At that time point, only 30% of the mutant conidia had undergone swelling, compared to 70% for the control strains. This defect in swelling, and subsequently in germination, could be explained by the higher susceptibility of *DprA*Δ conidia to the fungicidal effect of H_2O_2. To check the behavior of conidia challenged with host-derived reactive oxidant species, conidial survival was assessed after 36 h in the lungs of immunocompetent mice. Accordingly, the conidia of the *DprA*Δ and *DprA*Δ *DprB*Δ mutants were hypersensitive to killing by the lung phagocytes (Figure 3E). However, no difference was observed in the virulence of the strains in two experimental models of invasive aspergillosis (Figure S2).

DprB is involved in the osmotic stress response

When grown in the presence of sorbitol, *DprB*Δ (in contrast to *DprA*Δ mutants), displayed an abnormal colony morphology, which could be observed as from 0.5 M sorbitol (Figure 4A). *DprB*Δ colonies were restricted to forming a "central zone," in which conidiation would take place, with no "peripheral zone," in which the colony would normally expand by apical extension of the hyphae. With 1.5 M sorbitol, the *DprB*Δ colonies had one-half the diameter of the control strain colonies. Microscopic observation of the colony edges showed extensive branching (Figure 4B). The results were similar irrespective of the osmoticum (sorbitol, glycerol, mannitol, NaCl, KCl) or medium (Sabouraud, YPD, minimal medium, malt extract; unpublished data) used. Addition of 1.5 M sorbitol to the medium did not affect the germ tube emergence nor the germination kinetics of the mutant conidia compared with the control strains (unpublished data), indicating that osmostress did not affect the *DprB*Δ mutants at the conidial stage, but rather at later stages of development. When the wild-type strain was subjected to sorbitol concentrations ranging from 0 to 1 M, *DprB* transcript levels were up-regulated (Figure 4C). Regulation was dose-dependent, with an expression peak with 0.75 M sorbitol.

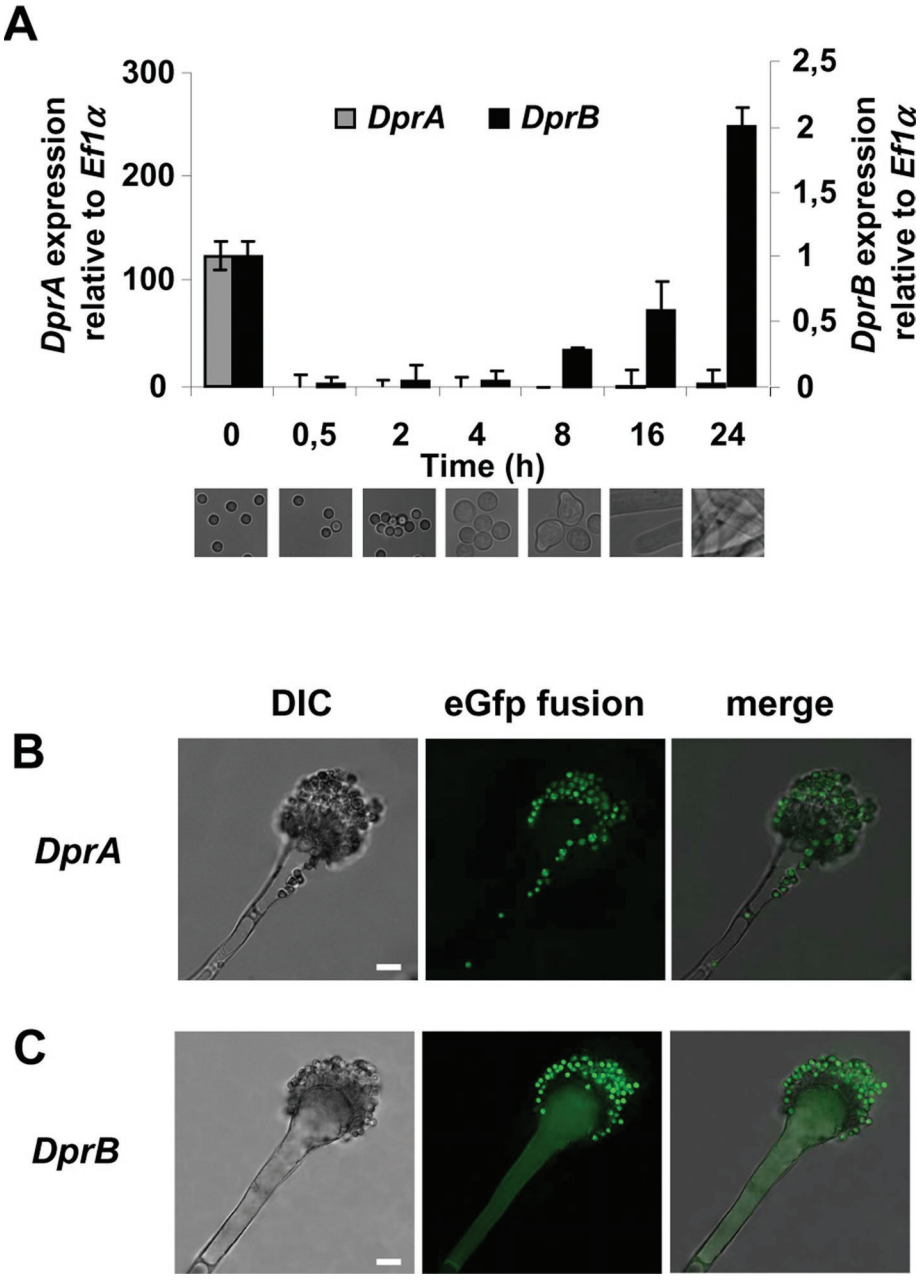

FIGURE 2: Expression of *DprA* and *DprB*. (A) Evaluation of the expression levels of *DprA* and *DprB* by real-time PCR. RNA was extracted from dormant conidia of the *AkuB* strain (0 h) and conidia that were incubated for 0.5, 2, 4, 8, 16, or 24 h in liquid YPD medium at 37°C, 150 rpm. An arbitrary value of 1.0 was attributed to the expression level of *DprB* at time 0. Data are from three independent experiments ± SE. Developmental stages corresponding to the selected time points are shown. (B) Assessment of *DprA* expression by *eGfp* fusion in hyphae undergoing conidiation (7 d at 25°C). Note that DprA-eGfp is present only in the conidia. (C) Same as (B), with *DprB* expression. Scale bars: 5 μm.

The *DprB*Δ mutant displays a pH-dependent phenotype that is PacC-related

Growth of the *DprB*Δ (but not of the *DprA*Δ) mutant was impaired at pH 7 and pH 9, but not at pH 5 (Figure 5A). At pH 9, after 72 h of growth, the *DprB*Δ colonies had one-half the diameter of the control strain colonies (unpublished data). Real-time PCR indicated *DprB* was expressed preferentially at neutral or alkaline pH (Figure 5B), consistent with the phenotype of the *DprB*Δ mutant. As seen in the assays under osmostress, germination of the conidia was not affected by pH stress (unpublished data). The PacC transcription factor controls pH-regulated genes in *Aspergillus* spp. (Tilburn *et al.*, 1995). A putative binding site of PacC (GCCAGG) was detected in the promoter of *DprB* at position −264. The binding of recombinant PacC to the DNA sequence was confirmed (Figures 5C and S3). In *Aspergillus* spp., PacC acts as both an activator of alkaline-expressed genes and a repressor of acid-expressed genes. Loss-of-function mutations of *PacC* (*PacC*$^{+/-}$) cause an acidity-mimicking phenotype and result in an increased expression of acid-expressed genes, and a reduced expression of alkaline-expressed genes. Gain-of-function, alkalinity-mimicking mutations of *PacC* (*PacC*^{c}) result in a phenotype opposite that of acidity-mimicking mutations. *DprB* expression was checked in the alkalinity-mimicking and in the acidity-mimicking strains (Amich *et al.*, 2009). In agreement with a positive regulation of *DprB* by PacC, the expression of *DprB* in the acidity-mimicking strain was similar to that in the wild-type, whereas it was overexpressed in the alkalinity-mimicking strain (Figure 5D).

DprA and DprB act downstream of the SakA MAPK

To gain insight into the signaling cascades involved in the recruitment of Dpr proteins, the expression of *Dpr* genes was assessed in signal transduction mutants from the mitogen-activated protein kinase (MAPK) pathways (*SakA*Δ, *MpkA*Δ, *MpkB*Δ, and *MpkC*Δ; Du *et al.*, 2006; Reyes *et al.*, 2006; Valiante *et al.*, 2008), the cyclic AMP (cAMP) signaling pathway (*AcyA*Δ, *PkaC1*Δ, and *PkaR*Δ; Liebmann *et al.*, 2003, 2004; Grosse *et al.*, 2008), and the calcium/calcineurin transduction pathway (*calA*Δ; da Silva Ferreira *et al.*, 2007). In contrast with other signaling mutants, *DprA* and *DprB* transcripts were not detected in the *SakA*Δ mutant (Figure 6, A and B), indicating DprA and DprB acted downstream of the stress-activated kinase (SAK) SakA MAPK cascade. In *A. fumigatus*, the SakA signaling pathway regulates the response to hyperosmotic and oxidative stress (Du *et al.*, 2006; Reyes *et al.*, 2006). An in silico comparative analysis was undertaken to identify targets of SakA that could link the MAPK to *Dpr* genes. In *Saccharomyces cerevisiae*, the SakA homologue Hog1 interacts with 4 transcription factors, Msn2, Msn4, Sko1, and Hot1 (Hohmann *et al.*, 2007). However, *A. fumigatus* lacked clear orthologues of these proteins (Bahn, 2008), indicating that other sets of transcription factors achieved stress-response regulation downstream of SakA. In *Schizosaccharomyces pombe*, two bZIP-type transcription factors, Atf1 and Pap1, intervene downstream of the SakA-related MAPK cascade in response to environmental stress signals (Toda *et al.*, 1991; Takeda *et al.*, 1995; Shiozaki and Russell,

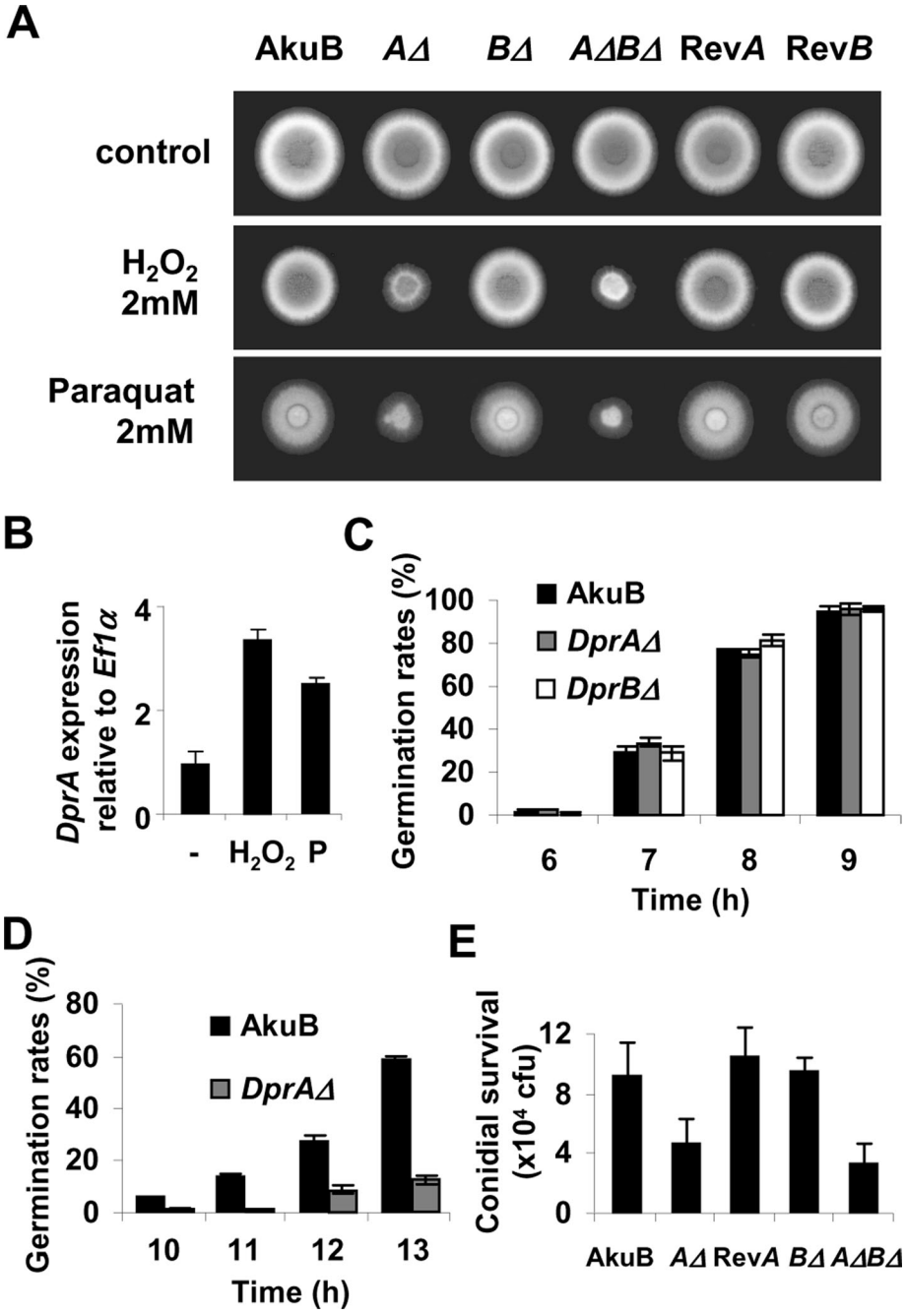

FIGURE 3: Oxidative stress-induced phenotype of *DprA*Δ mutants. (A) Growth of *Dpr*Δ mutants (*A*Δ, *B*Δ and *A*Δ*B*Δ) in the presence or absence of oxidative stress induced by H_2O_2 or paraquat, compared with the control strains (*AkuB*, or the revertant strains Rev*A* and Rev*B*). Conidia (10^5) were spotted on Sabouraud medium supplemented with 2 mM H_2O_2 or 2 mM paraquat (P). The plates were incubated at 37°C for 30 h. (B) Expression levels of *DprA* under oxidative stress. Overnight cultures of the *AkuB* strain were treated for 1 h with 2 mM H_2O_2 or 2 mM paraquat at 37°C 150 rpm, prior to RNA extraction. An arbitrary value of 1.0 was attributed to the expression level corresponding to the control without stress. Data are from three independent experiments ± SE. (C) Germination rates of the control (*AkuB*) and the *Dpr*Δ mutant conidia on Sabouraud medium at 37°C. Conidia undergoing germination were scored on a total of 100 conidia at 1 h intervals, in three independent experiments ± SE. (D) Same as (C), but in the presence of 2 mM H_2O_2. (E) Conidial killing in immunocompetent mice 36 h after intranasal infection. Conidial survival was evaluated by plating serial dilutions of bronchoalveolar lavages. Data are the mean value obtained with 3 mice ± SE.

1996; Gaits *et al.*, 1998; Toone *et al.*, 1998). *Aspergillus* spp. possess an *Atf1* and a *Pap1* homologue, and SakA was shown to interact with AtfA in *A. nidulans* (Lara-Rojas *et al.*, 2011). Mutant strains for *AtfA* (Afu3g11330) and *Yap1* (Afu6g09930) were constructed, and real-time PCR analysis showed that *DprA* expression was impaired in the *AtfA*Δ but not in the *Yap1*Δ mutant, indicating that *DprA* acted downstream of AtfA (Figure 6, A and B).

The expression of *DprA* was also affected in the adenylyl cyclase mutant *AcyA*Δ (Figure 6C). When the *AcyA*Δ strain was grown on medium supplemented with 25 mM cAMP, the wild-type expression level of *DprA* was restored, indicating regulation by the cAMP-related pathway. Consistent with this finding, putative cAMP-responsive element (CRE) sequences were found in the promoter of *DprA* at positions −142 (TGACGTAA) and −23 (GAACGTCA), to which a recombinant AtfA protein was able to bind (Figures 6D and S3).

DprA and *DprB* are induced by DTT

A major class of stress-protective molecules is represented by molecular chaperones. These molecules are essential for cells to prevent the aggregation of partially unfolded proteins. This requirement is increased when cells experience protein unfolding stresses. Upon treatment with dithiothreitol (DTT), an inducer of the unfolded-protein response, significant up-regulation of *DprA* and *DprB* expression was observed (Figure 7), suggesting a potent role of the corresponding proteins as molecular chaperones.

DprA and DprB fused to eGfp are associated with the cytosol and the peroxisomes

To check their subcellular localization, DprA and DprB were fused at their carboxy-terminal end to eGfp, under the control of their native promoters, in the respective mutant strains. The functionality of the constructs was checked by the restoration of the wild-type phenotype (unpublished data). Both DprA-eGfp and DprB-eGfp fusion proteins accumulated in the cytoplasm and in punctuate organelles. The labeled organelles did not stain with the membrane- and endocytosis-selective dye FM4–64 (Supplemental Movie S1; Fischer-Parton *et al.*, 2000). To test the hypothesis that the organelles might be peroxisomes, the DprA- and DprB-eGfp strains were transformed with a plasmid bearing a DsRed-serine-lysine-leucine (DsRed-SKL) fusion typical of the type 1 peroxisomal targeting sequence PTS1 (Ruprich-Robert *et al.*, 2002; Elleuche and Pöggeler, 2008). Colocalization of eGfp and DsRed showed that DprA and DprB were associated with peroxisomes (Figure 8 and Movie S2).

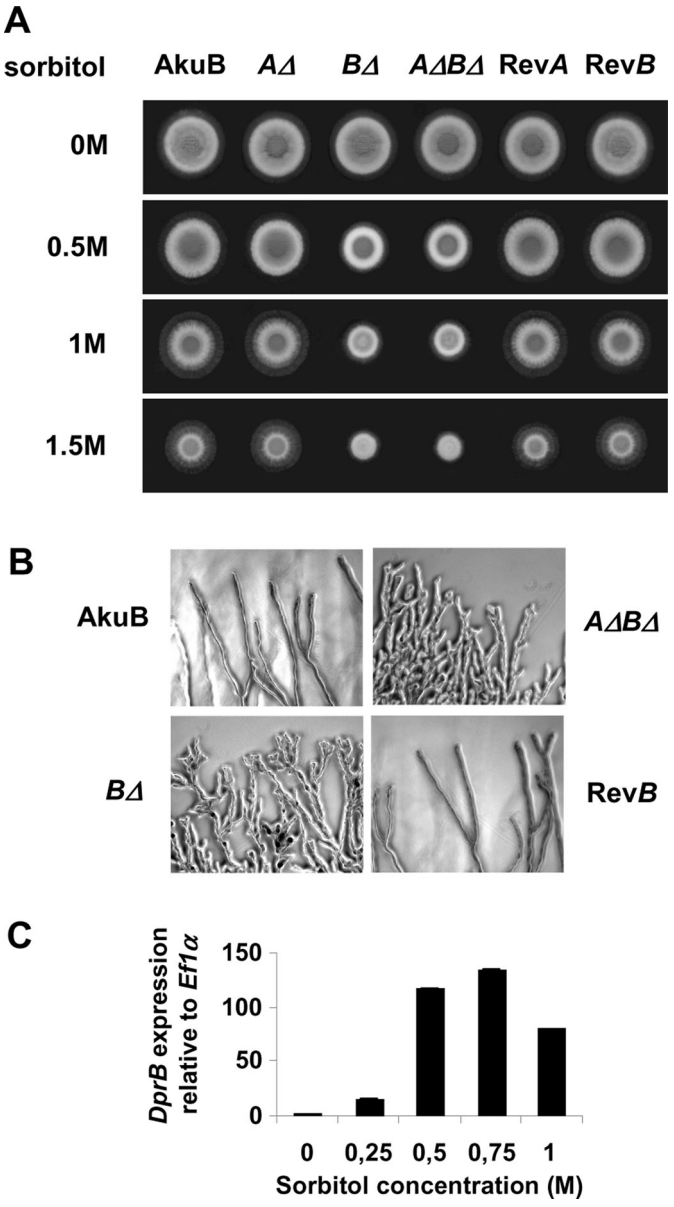

FIGURE 4: Osmotic stress-induced phenotype of *DprB*Δ mutants. (A) Growth of *Dpr*Δ mutants (*A*Δ, *B*Δ, and *A*Δ*B*Δ) in the presence or absence of osmotic stress induced by sorbitol, compared with the control strains (*AkuB*, or the revertant strains Rev*A* and Rev*B*). Conidia (10^5) were spotted on Sabouraud medium supplemented with 0, 0.5, 1.0, or 1.5 M sorbitol. The plates were incubated at 37°C for 30 h. (B) Microscopic observation of the colonies grown on solid Sabouraud supplemented with 0.5 M sorbitol. Note the hyperbranched mycelium of the *DprB*Δ and *DprA*Δ *DprB*Δ mutants, compared to the parental and revertant strains. (C) Expression levels of *DprB* under osmotic stress assessed by real-time PCR. Extracts are from overnight liquid cultures of the wild-type strain subjected to a range of sorbitol concentrations (0, 0.25, 0.5, 0.75, 1 M) for 30 min at 37°C. An arbitrary value of 1.0 was attributed to the expression level without addition of sorbitol. Data are from three independent experiments ± SE.

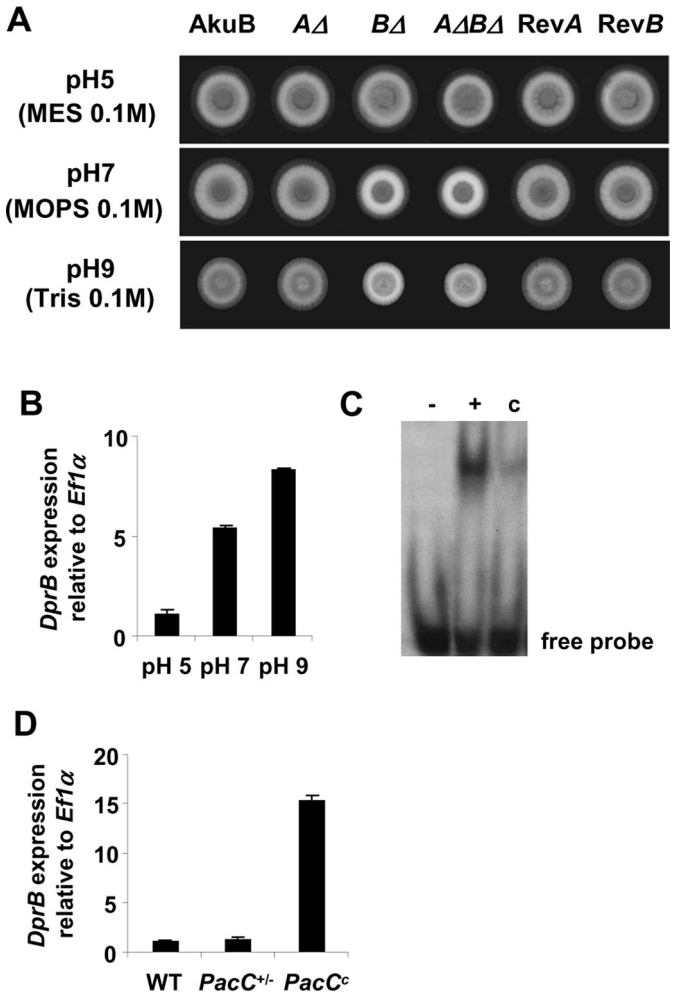

FIGURE 5: pH stress-induced phenotype of *DprB*Δ mutants. (A) Growth of *Dpr*Δ mutants (*A*Δ, *B*Δ, and *A*Δ*B*Δ) under pH stress, compared with the control strains (*AkuB*, or the revertant strains Rev*A* and Rev*B*). Conidia (10^5) were spotted on Sabouraud medium supplemented with 0.1 M MES, MOPS, or Tris and adjusted to pH 5, 7, or 9. The plates were incubated at 37°C for 30 h. (B) *DprB* expression according to extracellular pH. Real-time PCR was performed on extracts from overnight cultures shifted for 1 h to medium at pH 5, 7, or 9 at 37°C, 150 rpm. An arbitrary value of 1.0 was attributed to the expression level corresponding to pH 5. Data are from three independent experiments ± SE. (C) Gel-mobility shift assay using the GST::PacC (30–195*) protein produced in *Escherichia coli* and a 37 bp fragment of the *DprB* promoter. The ^{32}P-labeled DNA fragment (40 fmol) was incubated in the absence (−) or presence (+) of the purified GST::PacC protein (40 fmol). Unlabeled probe (4 pmol) was used as competitor (c) to check the specificity of the binding. (D) *DprB* expression in *PacC* mutants. Real-time PCR was performed on extracts from overnight cultures at pH 7 of the reference strain *AkuB*, the acid-mimicking strain *PacC*$^{+/-}$, and the basic-mimicking strain *PacC*^{c}. An arbitrary value of 1.0 was attributed to the expression level obtained with the wild-type strain (WT). Data are from three independent experiments ± SE.

*Dpr*Δ mutants have altered catalase and β-oxidation activities

Peroxisomes contain a large battery of enzymes that are important notably for oxygen species detoxification and β-oxidation of fatty acids. Catalases have a protective role against H_2O_2 and have been shown to be localized in peroxisomes (Schrader and Fahimi, 2006).

A. fumigatus possesses three catalase activities: CatA, which is produced exclusively in conidia, and Cat1 and Cat2, which are produced in the mycelium (Paris *et al.*, 2003). In an attempt to explain the higher sensitivity of *DprA*Δ conidia to H_2O_2, catalase activity was assessed in conidial protein extracts. As shown by in-gel detection, activity due to the conidial catalase CatA was reduced in the *DprA*Δ mutant (Figure 9A). This was not the case for the mycelial catalases

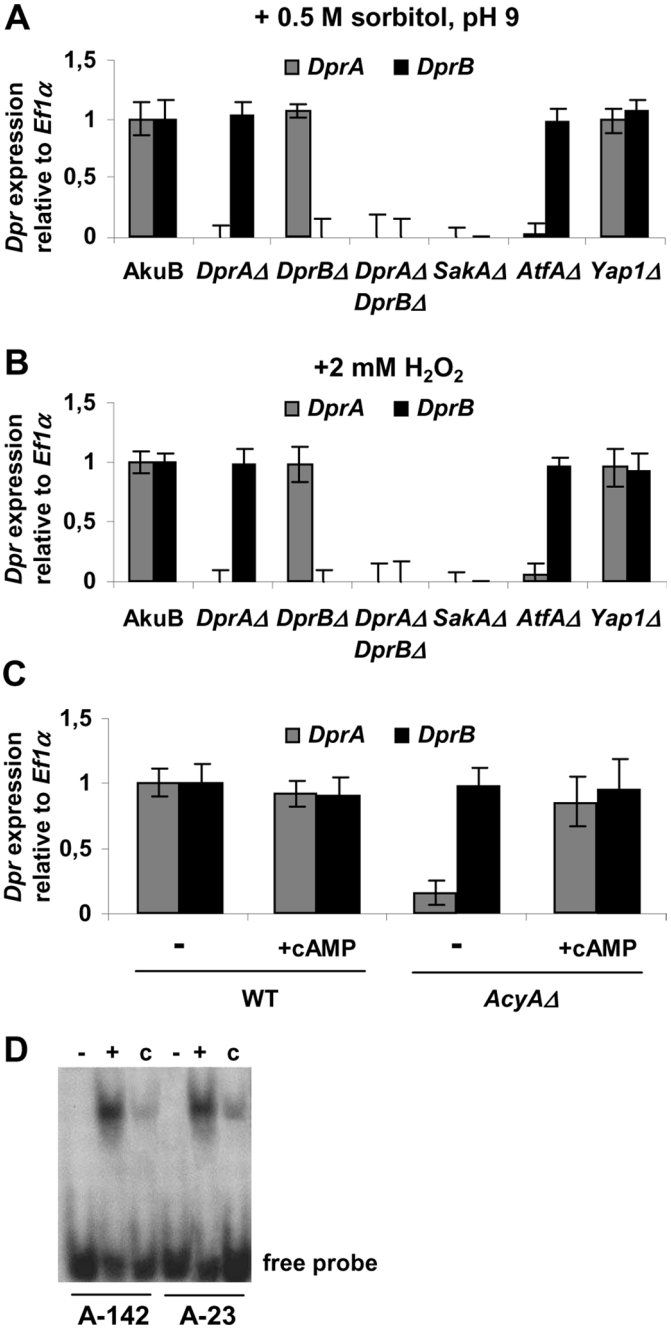

FIGURE 6: Signal transduction pathways involved in the expression of *Dpr* genes. (A) Expression of *Dpr* genes in *A. fumigatus* mutants under 0.5 M sorbitol, pH 9. Transcript levels of *DprA* and *DprB* were estimated by real-time PCR in the reference strain *AkuB*; the *Dpr* mutants *DprA*Δ, *DprB*Δ, *DprA*Δ *DprB*Δ; the MAPK mutant *SakA*Δ; and the transcription factor mutants *AtfA*Δ and *Yap1*Δ. An arbitrary value of 1.0 was attributed to the expression levels in the *AkuB* strain. (B) Same as (A), under 2 mM H_2O_2. (C) Expression of *Dpr* genes in the adenylyl cyclase mutant *AcyA*Δ, and the effects of adding 50 mM exogenous cAMP as determined by real-time PCR. An arbitrary value of 1.0 was attributed to the expression levels in the *AkuB* strain without treatment with cAMP. (D) Gel-mobility shift assay using the AtfA::6His recombinant protein and 37–base pair fragments from the *DprA* promoter (A-142 or A-23). The ^{32}P DNA fragments (40 fmol) were incubated in the absence (–) or presence (+) of the recombinant AtfA::6His protein (40 fmol). Unlabeled probe (4 pmol) was used as competitor (c) to check the binding specificity.

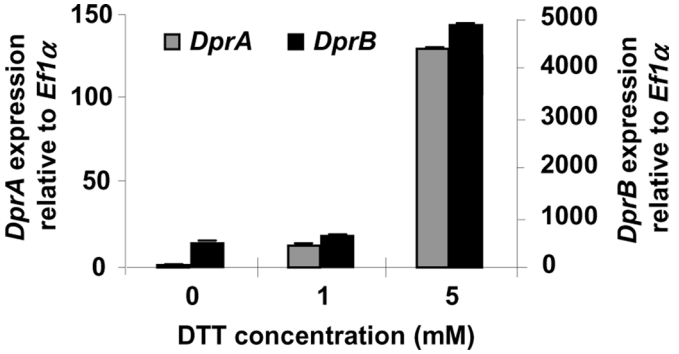

FIGURE 7: Expression of *DprA* and *DprB* upon treatment with DTT. Real-time PCR was performed on RNA extracted from overnight cultures of the *AkuB* strain treated for 1 h with 0, 1, or 5 mM DTT at 37°C, 150 rpm. An arbitrary value of 1.0 was attributed to the expression level of *DprA* in the absence of DTT. Data are from three independent experiments ± SE.

Cat1 and Cat2 when mycelial extracts were assessed (unpublished data).

Growth of the *DprB*Δ mutant was impaired when short-chain fatty acids (butyrate [C4], valerate [C5], hexanoate [C6]) were used as sole carbon sources (Figure 9B), but not with longer-chain fatty acids such as oleic acid (C18), or Tween 20, whose major component is lauric acid (C12; unpublished data). In *Aspergillus* spp., β-oxidation of short-chain fatty acids with odd-numbered carbons (notably valerate) requires the peroxisomal β-oxidation pathway (Hynes *et al.*, 2008). The two results are in agreement with a peroxisomal association of Dpr proteins.

DISCUSSION

Dpr genes encode specific stress-response proteins

While the *DprA*Δ mutant was impaired in growth on media supplemented with H_2O_2 or paraquat, the *DprB*Δ mutant growth was affected under hyperosmotic conditions or when pH was maintained at 7 or 9. Other stress conditions, e.g., extreme temperatures (storage at −20°C or 4°C, culture at 50°C), drug treatments (with caspofungin, Congo red, calcofluor white, or SDS), or high concentrations of metal ions (Fe^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, or Ni^{2+}) had no effect on the development of the mutants (unpublished data), indicating DprA and DprB were involved specifically but differently in oxidative, osmotic, and pH stress.

The protection effect exerted by Dpr proteins against stress was confirmed by overexpression studies of *DprA* and *DprB* that resulted in enhanced resistance to oxidative stress, and osmotic and pH stress, respectively (Figure S4). This is in good accordance with studies related to the overexpression of dehydrins and molecular chaperones, in either homologous or heterologous systems (Swire-Clark and Marcotte, 1999; Brini *et al.*, 2007; Montero-Barrientos *et al.*, 2008), although the stresses involved were different.

An excellent correlation was obtained between expression levels under stress and mutant phenotypes, suggesting that Dpr regulation was predominantly transcriptional. Gene expression was modulated transiently within the time frames described previously in filamentous fungi for the regulation of oxidative stress (30–60 min; Li *et al.*, 2008), osmotic stress (30–90 min; Han and Prade, 2002), and the pH response (30–120 min; Galindo *et al.*, 2007), indicating a genetic program involving *DprA* and *DprB* was rapidly switched on to reestablish homeostasis. However, posttranslational regulation cannot be excluded, since putative phosphorylation sites were detected (Figures 1 and S1).

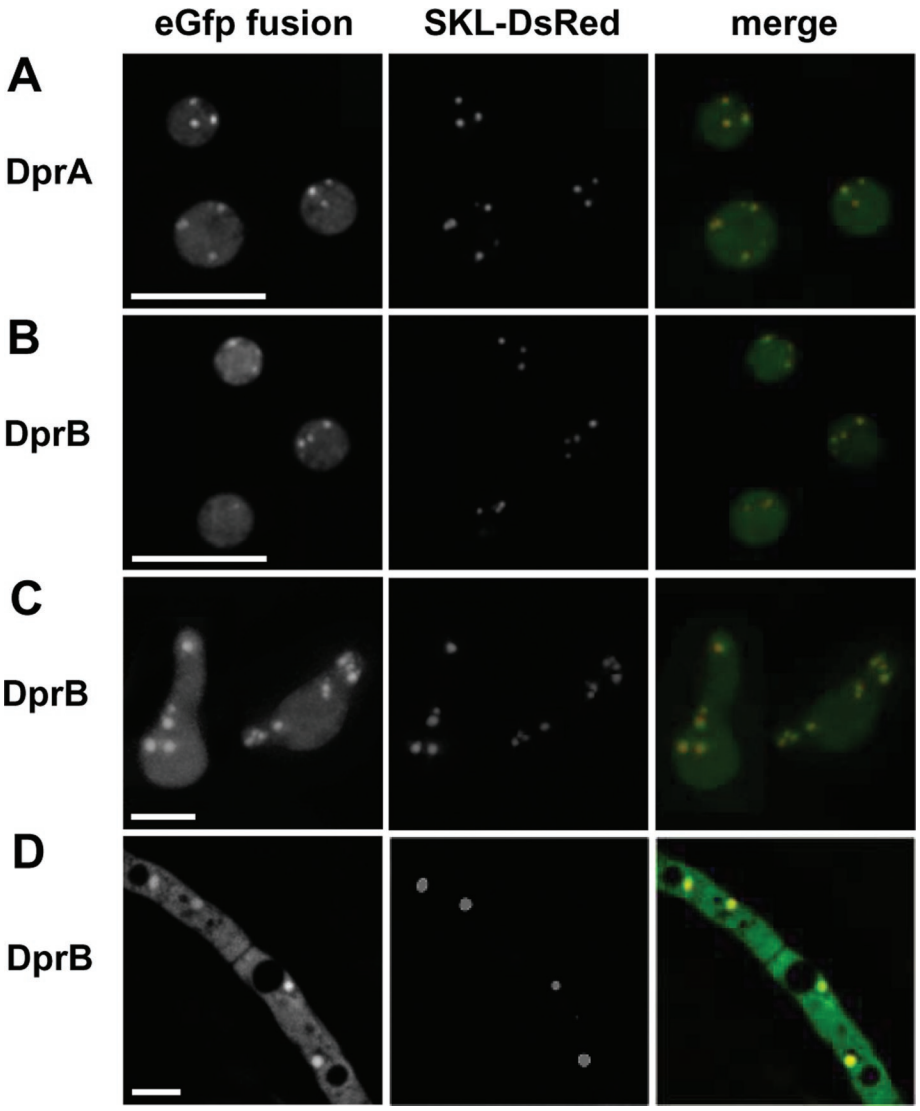

FIGURE 8: Subcellular localization of DprA and DprB using eGfp fusions. *DprA* and *DprB* were fused at their 3′ end to the coding sequence of *eGfp*. Expression was driven by the gene's own promoter in the corresponding *DprA*Δ and *DprB*Δ strains. Localization of DprA in dormant conidia (A), and localization of DprB in dormant conidia (B), in germinating conidia (C), and in hyphae (D). Scale bars: 5 μm

Signal transduction pathways involved in Dpr-related stress response

In this study, we showed that several pathways were involved in the regulation of *Dpr* genes. A hypothetical model is proposed in Figure 10. Common to *Dpr* gene regulation, the SakA pathway was known to control stress responses, either oxidative- (Nguyen *et al.*, 2000; Du *et al.*, 2006; Hagiwara *et al.*, 2009; Zhang *et al.*, 2009; Balazs *et al.*, 2010) or osmotic-related (Han and Prade, 2002; Liu *et al.*, 2008; Zhang *et al.*, 2009). Together with the cAMP-related pathway, SakA contributes to the oxidative stress response mediated by DprA. The AtfA transcription factor, known to intervene downstream of the cAMP-related pathway (Rehfuss *et al.*, 1991; Neely and Hoffman, 2000), is activated by SakA, most probably upon phosphorylation (Lara-Rojas *et al.*, 2011). The acquisition of DprA-related oxidative stress tolerance in the conidia is consistent with the fact that Pka- and AtfA-mediated oxidative stress tolerance in *Aspergillus* spp. is acquired specifically in conidia (Kawasaki *et al.*, 2002; Zhao *et al.*, 2006; Hagiwara *et al.*, 2008). Upon osmotic stress, transcription of *DprB* was dependent on the SakA-related pathway and on a hypothetical osmotic stress regulator, OsrA. The *SakA*Δ strain was unaffected by pH stress, whereas the *PacC* mutants were unaffected by osmostress (unpublished data), suggesting the two pathways converged independently on *DprB*.

The involvement of SakA in two distinct responses raises the question of signaling specificities. In *S. cerevisiae*, the Fus3- and the Kss1-related MAPK pathways converge on the Ste12 transcription factor to regulate mating or filamentation, respectively, in response to different stimuli. Signaling specificity is achieved by selective ubiquitination and sumoylation of Ste12 and its cofactor Tec1, leading to either degradation or protection of the respective transcription factors (Bruckner *et al.*, 2004; Chou *et al.*, 2008). Cross-activation of AtfA and OsrA in *A. fumigatus* could be prevented by a similar interplay between ubiquitination and sumoylation. This hypothesis is supported by the presence of two sumoylatable sites on AtfA (positions 399 and 474). Alternatively, dual phosphorylation of SakA by specific phosphatases could lead to the activation of different downstream effectors or OsrA could interact with SakA in the absence of phosphorylation, as shown for Hot1 (Saito and Tatebayashi, 2004).

Possible cellular function of Dpr proteins

Widely distributed in eukaryotes, intrinsically unstructured proteins (IUPs), which lack defined folding and three-dimensional structures, have high intramolecular flexibility (Tompa, 2002; Dyson and Wright, 2005). Dehydrins are IUPs that typically accumulate in plants in response to environmental stimuli with a dehydrative component (notably drought), salinity, and seed maturation (Battaglia *et al.*, 2008). They possess one or several K-segments (EKKGIMDKIKEKLPG) that are presumed to interact with key cellular regulators prone to misfolding, which allows their stabilization in a chaperone-like manner (Close, 1996; Kovacs *et al.*, 2008), thus precluding aggregation of proteins fatally damaged by stress. Tompa and Kovacs (2010) proposed that in virtue of their structural plasticity, IUPs may serve as potent chaperones. The possible role of Dpr proteins as molecular chaperones is supported by several lines of evidence: 1) *Dpr* genes are induced under stress and Dpr proteins subsequently exert a protective role against stress. 2) *DprA* and *DprB* expression is up-regulated upon addition of DTT, an inducer of protein misfolding. 3) The flexible structure of Dpr proteins, proline residues, and phosphorylation sites within the DPR domains are compatible with the physicochemical features expected in chaperones (Tompa and Kovacs, 2010). 4) Their association with the cytoplasm and the peroxisomes is consistent with the shuttling of peroxisomal precursors from the cytoplasm, where they are synthesized, to

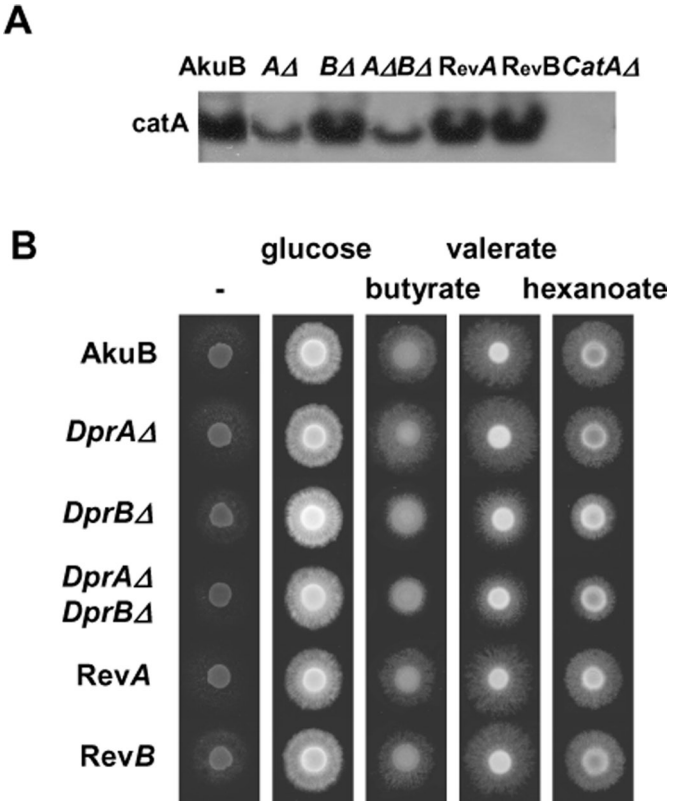

FIGURE 9: Effect of *Dpr*Δ mutation on peroxisomal functions. (A) In-gel detection of catalase activity. Each line was loaded with 30 µg total protein. (B) Growth on different carbon sources. The following carbon sources were added to modified minimal medium: 1% glucose, 10 mM butyrate, 10 mM valerate, 5 mM hexanoate. Growth was for 3 d at 37°C.

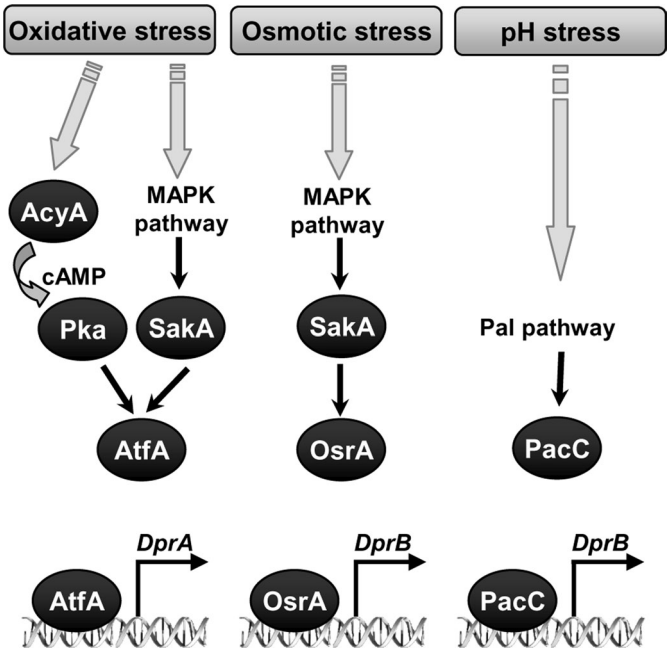

FIGURE 10: Proposed model for the regulation of *Dpr* genes. In response to oxidative stress, cAMP is synthesized by the adenylyl cyclase AcyA. Due to high levels of cAMP, protein kinase A (PKA) is activated. In parallel, oxidative stress also activates signal transduction via the SakA-related MAPK pathway. Both pathways converge on the bZIP transcription factor AtfA. Once activated, AtfA binds to the promoter of its target gene *DprA* to positively regulate its transcription. In response to osmotic stress, the signal is transduced via the SakA-related MAPK pathway to activate a hypothetical osmotic stress regulator (OsrA), leading to the transcription of *DprB*. When subjected to pH-related stress, the signal is transduced via the extracellular pH response pathway (Pal) pathway mediated by the zinc finger transcription factor PacC. PacC binds to the promoter of *DprB* to positively regulate its transcription.

the peroxisomes, where they are addressed (Götte *et al.*, 1998; Hettema *et al.*, 1998).

The absence of peroxisome-targeting signal (PTS) is in favor of a peripheral association of Dpr proteins with the peroxisomes. Other proteins, notably peroxins, which are known to be associated both with the cytosol and the peroxisomes, participate in peroxisomal protein import and do not exhibit any obvious PTS (Pex1 [Tamura *et al.*, 2006], Pex5 [van der Klei *et al.*, 1995], Pex7 [Marzioch *et al.*, 1994], Pex19 [Sacksteder *et al.*, 2000], Pex18/21 [Purdue *et al.*, 1998]). Such proteins are either "piggybacked" by partners that have a PTS (Glover *et al.*, 1994; Lee *et al.*, 1997; Klein *et al.*, 2002; Islinger *et al.*, 2009) or are cytosolic proteins associated with the outer sides of peroxisomes (Götte *et al.*, 1998 ; Hettema *et al.*, 1998; Purdue *et al.*, 1998).

Molecular chaperones associated with proteins being imported to the peroxisomes belong predominantly to the heat shock protein Hsp70/Hsp40 family, members of which were first identified due to their specific induction during the cellular response to stress conditions. Requirement of members of the chaperone Hsp70/Hsp40 family for peroxisomal matrix protein import was demonstrated (Walton *et al.*, 1994; Legakis and Terlecky, 2001). Other molecular chaperones involved in peroxisomal protein import include the peroxin Pex19 (Jones *et al.*, 2004; Vizeacoumar *et al.*, 2006) or the ankyrin repeat protein Ankr2A (Shen *et al.*, 2010).

In conclusion, our study uncovered novel proteins involved in the protection against stress. DprA and DprB likely act in the cell as molecular chaperones. Their identification from a conidial dormancy transcript profiling study points out the necessity for the fungus to exhibit a high level of stress resistance during the dormancy period.

MATERIALS AND METHODS

Strains and growth conditions

The *A. fumigatus* strains used in this study are listed in Supplemental Table S1. The strain *AkuB* (da Silva Ferreira *et al.*, 2006) was used as the recipient strain for genetic transformation, unless otherwise specified. All strains were maintained on 2% malt agar slants at 25°C. Conidia were recovered following a 7-d culture at 25°C, in sterile 0.05% Tween 20, and filtered through a 40 µm pore size filter (BD Falcon, le Pont de Claix, France). Recombinant proteins were produced in *Escherichia coli* BL21 (DE3) or *Pichia pastoris* GS115.

In silico analysis

Blast analyses were submitted to the National Center for Biotechnology Information facility (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Estimation of intrinsic protein disorder was done with PrDOS (http://prdos.hgc.jp/cgi-bin/top.cgi; Ishida and Kinoshita, 2007). Prediction of phosphorylation sites was done with NetPhos software (http://www.cbs.dtu.dk/services/NetPhos/). The hydrophobic cluster analysis (Gaboriaud *et al.*, 1987) plots were generated by the Mobyle Project's HCA server (http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA). Prediction of sumoylation

sites was performed using SUMOplot (http://www.abgent.com/tools/sumoplot).

Construction of *A. fumigatus* deletion, overexpression, and fusion strains

The cassettes used for the genetic transformation of *A. fumigatus* were generated by fusion PCR, or inverse PCR combined with conventional cloning, as described previously (Karababa *et al.*, 2006; Lamarre *et al.*, 2007). The primers are listed in Tables S2 and S3 and the constructs are schematized in Figures S5–S7. The deletion constructs consisted of a selection marker cassette encompassed by ~1 kb flanking regions of the target gene. Complementation cassettes were made out of 1 kb of the 5′ flanking region, followed by the wild-type coding sequence and the selection marker cassette. The resistance genes to hygromycin (*Hph*, amplified from pAN7.1; Punt and van den Hondel, 1992) or phleomycin (*Ble*, amplified from pAN8.1; Punt and van den Hondel, 1992) were used to construct the cassettes. The double mutant was constructed by deletion of *DprA* in the *DprB*Δ mutant. For the overexpression of *DprA* and *DprB*, the genomic sequences of *DprA* and *DprB* encompassed with 1 kb upstream and downstream regions were amplified and cloned into the pPTRII vector (Takara, Saint-Germain-en-Laye, France) in the *Hin*dIII and *Sma*I restriction sites, respectively. For the subcellular localization of DprA and DprB, eGfp (Thastrup *et al.*, 1999) was fused to the carboxy-terminal ends and the expression was driven by 1 kb of the gene native promoters. The mutant strains *DprA*Δ and *DprB*Δ were cotransformed with the eGfp fusion cassettes together with pAN8.1 or the pAN7.1 linearized with *Nde*I or *Hin*dIII, respectively. For colocalization experiments with the SKL-DsRed construct, the *A. fumigatus* strain CBS144–89 was used as the recipient strain for the eGfp fusions. The strains obtained were cotransformed with the plasmid bearing the linearized SKL-DsRed construct (Elleuche and Pöggeler, 2008), together with linearized pAN7.1 or pAN8.1, respectively. Transformation of *A. fumigatus* was carried out by conidial electroporation, as previously described (Lambou *et al.*, 2010). The transformants were verified by Southern blot analysis and real-time PCR.

Phenotype analyses

Phenotypic tests were carried out mostly in Sabouraud medium (2% glucose, 1% mycopeptone; Oxoid, Dardilly, France). When mentioned, YPD, 2% malt extract, or minimal medium (Cove, 1966) were used as alternatives. For pH response assays, Sabouraud medium was amended with 0.1 M Tris, MOPS, or HEPES, according to the desired buffer range. For carbon source assays, 10 mM ammonium chloride, instead of ammonium tartrate, was added to minimal medium. In-gel catalase activity and germination rates were determined as described previously (Paris *et al.*, 2003; Du *et al.*, 2006).

Production of recombinant proteins and gel-mobility shift assays

The plasmid pGEX-PacC (Tilburn *et al.*, 1995) was used to transform *E. coli*. The fusion protein was produced and purified on glutathione agarose (Sigma, Saint Quentin Fallavier, France) according to the manufacturer's instructions. The coding sequence of AtfA was amplified from *A. fumigatus AkuB* cDNA with the primers listed in Table S4, and cloned into the pKJ113 vector (Borg-von Zepelin *et al.*, 1998) between the *Xho*I and *Not*I restriction sites. Following transformation of *P. pastoris*, the recombinant 6×His-tagged protein was produced and purified on Probond resin (Invitrogen, Cergy Pontoise, France) as suggested by the manufacturer. The DNA sequences used as probes are listed in Table S4. Probe labeling and gel-mobility shift experiments were carried out as described by Herbert *et al.* (2002).

Real-time PCR

Total RNA (5 µg) was reverse-transcribed using the Superscript II Reverse Transcriptase kit (Invitrogen, Cergy Pontoise, France). Quantitative PCR assays were performed using 96-well optical plates (Thermo Scientific, Courtaboeuf, France) in an iCycler iQ (170–8740; Bio-Rad, Marnes-la-Coquette, France) according to Bio-Rad's manufacturer instructions. Each run was assayed in triplicate in a final volume of 20 µL containing the DNA template at an appropriate dilution, 1× ABsolute SYBR green Fluorescein (Thermo Scientific), and 100 mM each primer (Beacon Designer 4.0 software; Premier International Software, Palo Alto, CA; Table S5). The cycling program was 95°C for 15 min, 40 cycles of 95°C for 30 s, and 55°C for 30 s. Amplification of one single specific target DNA was checked with a melting curve analysis at the end of the PCR (+0.5°C ramping for 10 s, from 55°C to 95°C). The generated data were then analyzed with Optical Systems Software 3.1 (Bio-Rad). The expression ratios were normalized to *Ef1*α expression, and calculated according to the ΔΔCt method (Livak and Schmittgen, 2001). To verify the absence of genomic DNA contamination, negative control templates in which reverse transcriptase was omitted were used for each gene set.

Live-cell imaging

For all microscopy experiments, *A. fumigatus* was cultured in Lab-Tek 8-well chamber-slides (Nalge Nunc International, Roskilde, France) containing Vogel's minimal medium N (Vogel, 1964). Wide-field epifluorescence imaging was performed on an inverted Nikon TE2000 microscope equipped with a Hamamatsu Orca ER CCD camera and a pE-2 LED excitation system (CoolLED) as the epifluorescence light source. Exposure times ranged from 200 to 400 ms; z-stacks of optical sections at 0.5 µm steps. Images were processed through 10 iterative deconvolutions using AutoQuant X software (Media Cybernetics, Bethesda, MD). For time-course experiments, images were acquired every 30 s for 10 min. Movies were generated and manipulated using ImageJ software (http://rsbweb.nih.gov/ij/). For membrane staining, germlings were incubated in 10 µM FM4–64 (Molecular Probes, Cergy Pontoise, France) for 30 min at 30°C.

In vivo experiments

Conidial survival assays in mouse lungs were carried out with inoculation of 5×10^8 conidia as described by Lambou *et al.* (2010). The virulence of the strains was tested in two models of invasive aspergillosis: cyclophosphamide and cortisone acetate-treated mice (Smith *et al.*, 1994) and *Galleria mellonella* waxworms (Renwick *et al.*, 2006). The virulence in immuno-suppressed mice was as described by Mouyna *et al.* (2010), except that the inoculation was with 1.5×10^5 conidia, and the cyclophosphamide dosage was 150 mg/kg. For the *G. mellonella* experiments, waxmoth (about 0.3–0.4 g in body weight) in the final larval stage (RJ Mous Livebait WOF, Netherlands) was used (10 per strain). 1.5×10^4 spores in phosphate-buffered saline (PBS) per larvae were used for injection into the hemocoel of 10 larvae per strain. The waxmoth larvae were then incubated at 37°C for up to 10 d, and survival was recorded daily.

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